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A Method for Determining Proteinase Activity

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A METHOD FOR DETERMINING PROTEINASE ACTIVITY

by

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INTRODUCTION

Enzymes play a very important role during the ripening process of the cheese. Enzymes are important in producing the body, texture, and flavor of the cheese. The breakdown of carbohydrates, fats, and proteins by added enzymes into different end products during the ripening of the cheese aids the process and may reduce time. The characteristic flavors of different varieties of cheeses are influenced by enzyme action. Some enzyme like proteinases can be used to improve the body, texture, and the flavor of the cheddar cheese. Addition of these enzymes during cheddar cheese making may shorten the ripening period. In order to use some specific amount of the enzyme to bring about desirable changes, it is desirable to determine the activity of that enzyme.

A variety of methods have been used to determine the activity of proteolytic enzymes. The methods employ different substrates, both natural and synthetic. Even with the same substrate, the procedures for conducting the test often vary. The A.O.A.C. (Fuld Gross Method), (1) which employs Hammersten's casein as the substrate, has been used quite extensively. In one case, hemoglobin is used as the substrate (2,3). Colormetric apparatus can be used to measure the partial hydrolysis of its protein.

Activation of the enzyme apparently takes place during the cheese making process and during the storage to produce cheese with a softer and more elastic body.

REVIEW OF LITERATURE

In cheese ripening the breakdown of casein represents one of the most important changes. Ichiro, Ishibara (20) and W. Keller (22) studied the breakdown of casein into its different products. The breakdown has been attributed at one time or another to the natural proteinase in milk, to the proteinases in rennet or to the proteinases of microbial origin. It was first suggested by Babcock and Russell (4) and Babcock et al. (5) that an inherent milk proteinase is the most important proteolytic agent in the cheese ripening. Later work on the variation of rennet levels in cheese making caused Babcock, et al. (5) to revise their early views. The final conclusion was that rennet is the most important proteinase source in cheese, its protein digestive activity being due to the action of pepsin present in the rennet as an impurity. Some workers (6, 9, 18, 23, 33, 34, 49) studied the effect of rennin on milk in relation to coagulation. Sherwood (35), however reported that the use of pepsin instead of rennet in cheddar cheese resulted in 40-50 percent less protein degradation than took place in normal rennet control cheeses. Several workers (8, 13, 14, 16, 19, 21, 36, 37, 40, 43, 46) were able to isolate proteinase from bacteria and able to study some of their effects on different cheeses.

Peterson, Johnson, and Price (31, 32) were able to determine the proteinase content of the cheddar cheese during making and ripening. A. E. Axetrod (3) and C. J. Martin (26) studied the proteolytic enzyme system of skin in relation to its purification and its activity. Several workers (11, 15, 17, 24, 38, 46) were able to study plant proteinases and their activity in the crystalline stage. W. J. Ellis (15) J. R. Kimmel and E. L. Smith were able to prepare crystalline papain of more purity. While A. Walri (46) was able to prepare crystalline ficin from the fig latex. Zettle (50) studied the effect of some salts on milk clotting by heat.

During the past several years, one of the major problems to be dealt with in the dairy industry has been concerned with the ripening of the cheddar cheese. In an effort to attack this problem from enzymatic view point, it was first necessary to determine the proteinase activity and then use the enzymes in the cheddar cheese manufacture. Addition of these enzymes during the cheese making may shorten the ripening period and improve the body texture and flavor of the cheddar cheese.

It is believed that the activity of the proteinase enzyme depends upon the structural configeration of that particular enzyme (28) specificity and activity also depend upon the constitutional properties common to a group of homospecific enxymes that might account for the similarity of their specificity requirements. At present, such questions cannot be answered since our present knowledge

of the constitutional details of proteolytic enzyme is almost nil. There is only one constitutional property that we know to be common to many proteolytic enzymes, and that has frequently been discussed in connection with their classification. It is a fact that many proteolytic enzymes are active only after they have been activated by HCN or sulfydryl compounds. Others are active when activated by metals. The activation by HCN and sulfydryl compounds is frequently and, according to Nord and Workman, (28) erroneously regarded as a reduction of the general type described schematically in table 1.

Table 1. The activation of intracellular enzymes

Oxidation-Reduction Theory		
2 (Enz. SH)	Oxidation	Enz. S-S Enz.
Proteolytically	Reduction	Proteolytically

On the other hand, a group of enzyme chemists have advanced the hypothesis that all proteolytic enzymes have a dualistic nature in that they consist of a colloidal protein acting as a carrier and a specific active part of unknown chemical nature. It is known from the experiments of Johnson (20) that the activity of the intestinal enzyme the hydrolyses L-Leucylglycine increases considerably on the addition of manganese or magnesium salts. The enzyme is also known to be inhibited by HCN or H_2S. Nord and Workman (28) pointed out that, these properties may be taken as indications

that the active enzyme is a dissociable metal protein compound. Indeed, it has recently been observed that on dialysis of the active enzyme preparation is obtained which is completely inactive towards L-Leucylglycine but which regains a high activity on addition of manganese or magnesium salts. This reactivation is the time reaction and the degree of the final activity depends upon the concentration of the metal ions added.

The activation of the intracellular enzymes of plants and animals by HCN and sulfhydryl compounds is complicated by the fact that these enzymes are almost always accompanied by sulphur containing compounds which are sometimes referred to as natural activators (43). Recently papain and cathepsin preparations have been obtained that contain no natural activators (24). Such purified papain preparation was found to be completely inactive towards the substrate benzoylarginine amide and it remained inactive after HCN had been added. There are some enzymes like purified papain trypsinase which is inactive and is not activated by HCN and which is known as papain trypsinase may be activated by H_2S (28). When the H_2S is subsequently removed in vacuo, a second inactive form papain B trypsinase is obtained. B. trypsinase can be activated by HCN.

The reaction can be represented as follows:

$$\underset{\text{(inactive)}}{\text{papain trypsinase}} \underset{\text{Oxidation}}{\overset{\text{Reduction}}{\rightleftharpoons}} \underset{\text{(inactive)}}{\text{B papain trypsinase}} \underset{\text{Evacuation}}{\overset{H_2S}{\rightleftharpoons}} \underset{\text{(active)}}{H_2S\text{-B papain trypsinase}}$$

According to Colowick and Kaplan (10) the plant proteinases are broadly classified in table 2.

Table 2. Properties of plant proteinases (10)

Enzyme	Common name of plant	Genus and species	Source of material	Crystal-line form	Isoelectric PH	Stability in acid or alkali	Heat Stability	pH of optimum
Papain	Papaya	Carica Papaya	Latex of green fruit	Needles Hexagonal plates	9	Unstable below pH 2.5 and above 12	Half life 56 min. at 75° C	7 - 7.5
Chymo-papain	Papaya	Carica Papya	Latex of green fruit	Needles plates	-	Stable at pH 2	-	7
Ficin	Fig	Ficus Carica Glabrata Coliaria	Latex	Hexagonal plates	5	Stable at pH 2	-	7
Mexicain	Cuagua-yote	Pileus Mexicanus	Leaves fruit	Lanceolate plates	-	Stable at pH 8	-	-
Asclepain	Milk-weed	Asciepias Speciosa Mexicana Cyriaca	Latex roots	Rectangular plates	3.1	Unstable in acid or alkali	Asclepain 78 min. at 60°	7 - 7.5
Bromelin	Pine-apple	Anana Satira	Fruit leaves	-----	-	Unstable at PH 2.3	21-5 min. at 60°	6 - 7
Pingui-nain	Maya	Bromelia Pinguin	Fruit	-----	-	-	-	3
Taberna Mountanin		Taberna-montana Grandiflora	Sap fruit	-----	-	-	-	5 - 6

Table 2. (Continued)

Enzyme	Common name of plant	Genus and species	Source of material	Crystal-line form	isoelectric pH	Stability in acid or alkali	Heat stability	pH of optimum
Soyin	Soya bean	Soya Hispidus	Germin-ated beans	-	-	-	-	6 - 8
Solanain	Horse-nettle	Solanum Elaeagni-folium	Fruit	-	-	Stable in diluted alkali	51 min. at 75°	8.5 weak 8
Euphor-bain	Caper Spurge	Euphorbia Lathyris	Latex	-	-	-	-	6 Strong
Hurain	Jabillo	Huracrepi-tans	Sap	-	-	4-5 stable in diluted alkali	-	5 Weak
Pomiferin	Osage Orange	Maclura Pomifera	Fruit	-	-	-	-	6.5
Arachain	Peanut	Arachis Hypagen	Seed	-	-	-	Destroys above 40°	6 - 7.5

The structure of the activated enzyme agrees with the theory of the dualistic nature of proteolytic enzymes. However, specificity studies do not support the claim that the protein part of the dualistic enzyme is merely a colloidal carrier for another and supposed to be active.

There are many methods available which can be used for determining proteinase activity. The methods like Van Slyke reaction method, formal titration. Ninhydrin colorimetric test can be used in determining proteinase activity. It is the purpose of this study to compare some methods like the A.O.A.C. (Fuld Gross Method) (1), Curd Tension Method, Clotting Method, which are now available and then modify a suitable one. The A.O.A.C. specifies the Fuld Gross Method which is quite elaborate and time consuming. The method is based on determining the rate of protein hydrolysis.

EXPERIMENTAL PROCEDURE

In this project two different types of enzymes, animal proteinase, and vegetable proteinase were used. An inactive animal enzyme the so called proenzyme or zymogen lot No. D. 870-156-1 was obtained from the "Armour Laboratories" and mixase C an active enzyme which contained mixture of enxymes Lot No. 530-295-1 obtained from the same laboratories. The proenzyme or zymogen was activated by the addition of small amount of an active enxyme mixase C.

The vegetable proteinase "p" which was an active enzyme Lot No. 20208 obtained from "Paul Lewis Laboratories." Since the vegetable proteinase was active it was used directly. The source of the vegetable proteinas was from the latex of the fruit of papaya (Carica papaya).

The following three methods were compared for enzyme activity and then the suitable one was modified for practical purposes.

1. Fuld Gross Method.
2. Curd Tension Method.
3. Clotting Method.

Fuld Gross Method

Method

Fuld Gross Method outlined by A.O.A.C. is as follows:

Inactivated. If the enzyme preparation is solid, finely divide it by grinding to a smooth paste in a small morter with a little freshly boiled cold water. Then suspend in cold boiled water. After 5-10 minutes centrifuge and discard sediment.

Activated. Proceed as directed in (a) but use half standard H_2S - H_2O instead of boiled water. After centrifuging, incubate enzyme solution at 40° C. for one hour to complete activation.

Reagents

Casein solution. Make 6 percent solution of Hammerstein's casein solution using 60 grams of casein with a little water in morter and gradually adding 60 ml. or 1NNaoH and water until volume totals 1 liter. Heat viscous solution for 30 minutes in the bath of boiling water; cool and filter if necessary.

Buffer solution. Prepare 0.2 M. monosodium citrate solution by partial neutralization of citric acid with NACH.

Titrating solution: 0.1 N. alcoholic KOH.

Indicator. 1 percent alcoholic thymopthalein solution.

Determination

Place 10 ml. of the casein solution and small charge of 4 mm. diameter glass beads in each of several 125 ml. glass-stoppered bottles and bring bottles and contents to 40° C. Add desired volume of prepared enzyme solution

but do not use more than 4 ml. If the quantity is insufficient, prepare a more concentrated solution of enzyme. Add immediately exactly 3 ml. of buffer solution in constant-temperature water bath at 40° C. Incubate mixture 20 minutes at 40° C., counting time from adding of buffer. Add 1 ml. of the indicator and begin titrating with the titrating solution. As soon as deep blue color appears, shake the bottle until the color is discharged or ppt. is completely dissolved. (It is usually best to add the alkali in doses of ca. 0.5 ml. at a time.) When all ppted. casein has been brought into solution, transfer contents of bottle to 400-500 ml. flask and rinse out bottle 2-3 times with alcohol, using total of 25 ml. for this purpose. Add a sufficient amount of the KOH solution to restore blue color, then add 175 ml. of boiling alcohol. Carefully add more KOH solution until pale but distant blue color persists in solution. Make control titration exactly as described, but do it immediately after addition of the buffer and therefore without any incubation time. Differences between titration of undigested sample and that of digested sample is a measure of proteolytic activation of the enzyme.

Calculation of proteinase unit

For smaller quantities of enzyme extent of hydrolysis determined by titration described is a straight line fraction of quantities of papain used. For accurate work

determination, draw a straight line by making several titrations with different quantities of enzyme. If quantities of papain used are too large, the straight line relationship will no longer hold. If they are too small, determinations will be inaccurate. Quantities of enzymes giving titration differences of 0.6 - 1.2 ml of 0.1 N. KOH are recommended. A unit of papain may be considered to be quantity of enzyme that produces under conditions outlined. Titration differences of 1 ml. of 0.1 N. KOH are determined either graphically or arithmetically. The value of the original preparation is then expressed in units/mg. as mg. of papain necessary to make one unit.

Curd Tension Method

One hundred cc. of milk of good quality was placed in glass jars as used for curd tension measurements. Samples were placed in a constant temperature water bath at 96° F.

To 100 cc. of milk, 1 cc. of solution of enzyme to be tested was added. This was followed by addition of 1 cc. of rennet solution made by mixing 9 cc. of rennet extract with 91 cc. of cold water.

After 20 minutes, the curd was tested with a curdmeter. Three g of active proteinase along with 1.5 g of inactive proteinase were dissolved in 50 cc. of distilled water and the solution was then centrifuged at 1000 rpm for 10 minutes and supernatent solution was used for the test.

Clotting Method

Although the milk clotting action of the proteinase has been recognized for many years, this property as the enzyme has received little quantitative attention.

The method was based upon the principle of clotting the ca-caseinate in a certain interval of time. Add a suitable amount of enzyme to be tested in 200 ml. milk. The milk containing the enzyme was placed in a 250 cc. graduated flask with a hole near the bottom in which was inserted a capillary glass tubing which is bent in such a manner as to prevent the milk flowing through the tube to drip slowly on a piece of black bakelite so the clot could be easily detected and the time was recorded.

The enzyme solution was added to the milk immediately before the addition of rennet. On the other hand, when fully activated enzyme was added to the milk, the rate of digestion or breakdown of the curd is sometimes so rapid that much of the casein is lost in the whey. Futhermore, an active enxyme produced a curd which was soft and flocculent.

RESULTS AND DISCUSSION

Different concentrations of animal proteinase was used and the average activity in terms of units/mg was measured by A.O.A.C. method. It was found that increase in concentration of the enzyme increases the average activity in terms of units/mg. Figure 1 of enzyme concentration was plotted against activity units/mg was a straight line indicating enzyme activity increases with increase in enzyme concentration.

Table 3. Enzyme concentrations and the average activity. (A.O.A.C. Method)

Concentration of enzyme (ml.)	Average activity Units/mg.
0.1	0.21
0.2	0.45
0.3	0.60
0.4	0.80
0.5	0.95
0.6	1.20
0.7	1.32
0.8	1.50
0.9	1.70
1.0	1.85

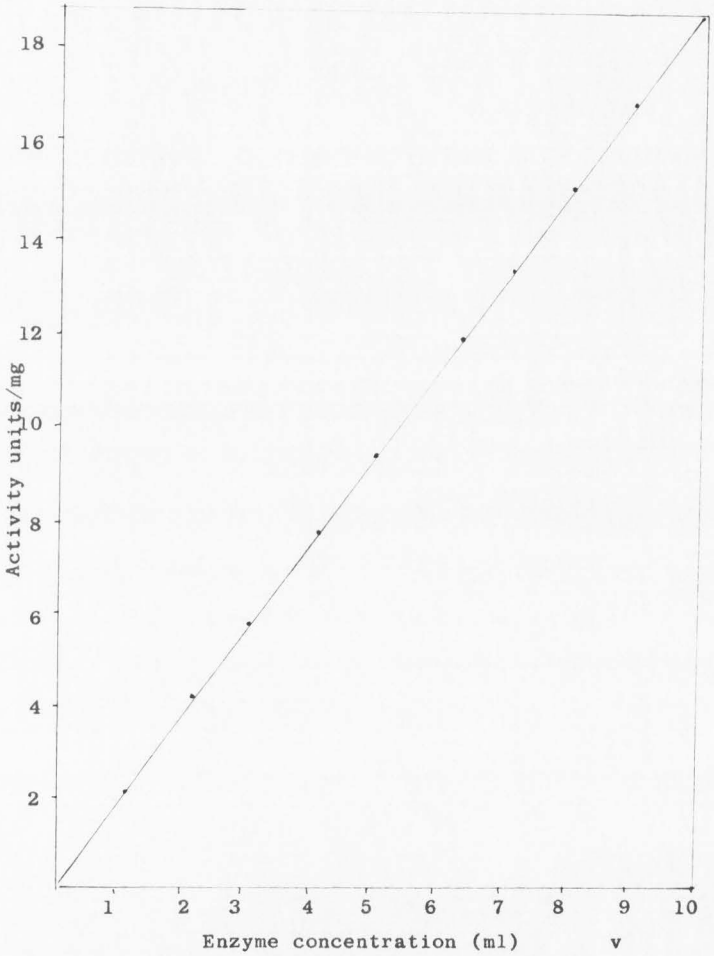


Figure 1. Relationship between enzyme concentrations and its activity (A.O.A.C. Method) (Animal proteinase)

Table 4. Curd tension method: relationship between enzyme concentration and the clotting time

Enzyme concentration in ml.	Time of clotting in min.
0.1	2.30
0.2	2.17
0.3	1.38
0.4	1.30
0.5	1.13
0.6	0.95
0.7	0.80
0.8	0.65
0.9	0.57
1.0	0.47

Different concentrations of proteinase was used for clotting the milk, but the curd was soft so that the curd-meter did not give any significant results.

Table 5. Clotting method: relationship between enzyme concentration and the clotting time

Enzyme concentration in ml.	Time of clotting in min.
-.1	2.30
0.2	2.17
0.3	1.38
0.4	1.30
0.5	1.13
0.6	0.95
0.7	0.80
0.8	0.65
0.9	0.57
1.0	0.47

Different concentrations of proteinase was used and the time of clotting was recorded. It gave some significant results. Then it was thought to modify the clotting method.

Modified Clotting Method

Different combinations of inactive on active enzymes were tried. The best results were obtained. The following combination was used:

0.7 grms. of unactive enzyme

0.3 grms of Mixase C (active enzyme)

The above enzymes were dissolved in 100 cc. of distilled water. Lower concentrations than this of active enzyme increased the clotting time; while if the higher concentrations than the above were used, then too soft curd and more proteolysis took place.

Preparation of enzyme solution

0.7 grms. of unactive proteinase DH 70-150-1 was accurately weighed out and it was then dissolved in 100 cc. of distilled water. Then 0.3 grms. of mixase C, an active proteinase, was dissolved in the same solution. The solution was then centrifuged at 1000 rpm for 15 minutes, and the sediment was removed. The supernatent enzyme solution was taken for the test.

The milk was heated to 37^{o} C. before using the enzyme. It seems that at the concentration of 4 - 5 mg., it gives a good curd. The body and texture of the curd is also quite good.

Kinetics of the clotting process

Except for very small concentrations of proteinase, the time required for clotting is inversely proportional

to the amount of proteinase present.

$$\text{Clotting time} \propto \frac{1}{\text{Amount of proteinase present}}$$

The relationship between the clotting time and the enzyme concentration is therefore a straight line as shown in figure 1.

Table 6. Effect of variation of animal proteinase enzyme

Amount of milk	Amount of enzyme	Time of clotting in min.	Type of curd	E = K/T or E = ET
10 cc.	15 mg.	1.3	too soft	19.5
10 cc.	12 mg.	1.15	too soft	13.80
10 cc.	10 mg.	1.37	too soft	13.70
10 cc.	9 mg.	1.50	soft curd	13.50
10 cc.	8 mg.	1.54	soft curd	12.32
10 cc.	7 mg.	2.0	soft curd	14.00
10 cc.	6 mg.	2.15	soft curd	12.9
10 cc.	5 mg.	2.20	good curd	11.00
10 cc.	4 mg.	2.25	good curd	9.00
10 cc.	3 mg.	2.50	semi-hard	7.20
10 cc.	2 mg.	3.15	semi-hard	6.30
10 cc.	1 mg.	4.10	semi-hard	4.10

Where E is the weight of the enzyme in milligrams and and T is the time in minutes and K is constant

$$E = K/T \text{ - - - - - - - - - - - (1)}$$

It follows from the equation (1) that:

$E = K$ when $T = 1$, therefore the activity per mg. $(\frac{1}{E})$ is $\frac{1}{K}$.

At low concentrations of proteinase, however, the relationship no longer holds. The time required for coagulation by a small dose of enzyme is much longer than

would be expected from the foregoing and the system behaves as though a part of the enzyme did not take part in the clotting reaction. Experiments have shown, however, that the amount of proteinase too small to clot the milk within a reasonable time, nevertheless, has the same effect on the system because such treatment of milk reduces the clotting time observed when an adequate quantity of enzyme is subsequently added.

If C denotes the amount of enzyme removed from the action during clotting, then available enzyme is E - C. C can be determined by drawing an intercept on the E axis when $\frac{1}{T}$ is plotted against E, and the previous expression (equation 1) becomes

$$(E - C)T = K$$

This accurately describes the relation between the time and the enzyme concentrations over the range of our experiments.

While the clotting with rennin is represented by the expression

$$E\ (T - X) = K,$$

X is probably the time lag of clotting after proteolysis has reached the requisite stage.

Too low concentration of rennin in presence of proteinase gave semi-hard curd, as in table 7, while too high concentrations of rennin the the presence of proteinase gave very soft curd and the separation of whey. The optimum concentration of rennin is between 0.3 - 0.4 ml.

Table 7. Effect of variation of rennet extract on the animal proteinase activity[a]

Amount of milk in ml.	Amount of rennet solution	Amount of enzyme	Time of clotting (in min.)	Type of curd
10 ml.	0.1 ml.	5 mg.	1.58	semi-hard
10 ml.	0.2 ml.	5 mg.	1.52	semi-hard
10 ml.	0.3 ml.	5 mg.	1.43	good curd
10 ml.	0.4 ml.	5 mg.	1.37	good curd
10 ml.	0.5 ml.	5 mg.	1.20	soft curd
10 ml.	0.6 ml.	5 mg.	1.17	soft curd
10 ml.	0.7 ml.	5 mg.	1.70	very soft
10 ml.	0.8 ml.	5 mg.	0.71	very soft
10 ml.	0.9 ml.	5 mg.	0.68	very soft
10 ml.	1.0 ml.	5 mg.	0.52	very soft and separation of whey

[a]9 cc. of the rennet extract was dissolved in 91 cc. of the distilled water.

Effect of variation of S.N.F. in milk

It is also interesting to observe the effect of different concentrations of solids non-fat on the proteinase activity. The milk samples were first analysed by the Majonnier method.

From the table 9 and the figure 3 it concluded that increased concentrations of S.N.F. decreases the clotting time and hence increases the proteinase activity. At very high concentrations of S.N.F., the activity of the proteinase decreases.

Mechanism of proteinase action on casein

Concerning the mechanism of the reaction between the

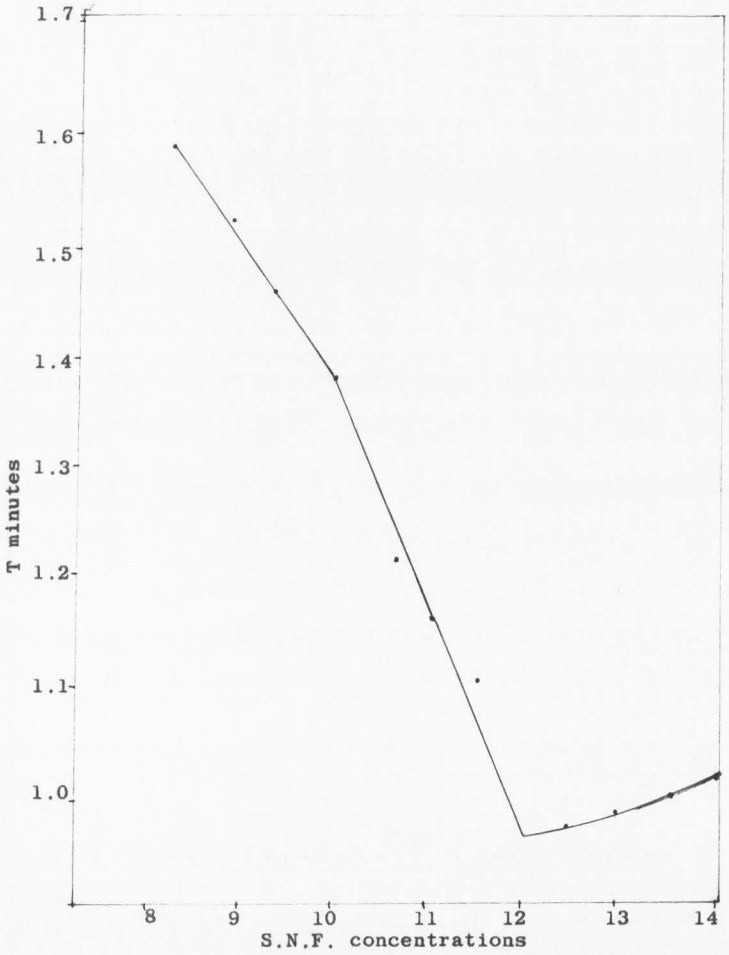


Figure 2. Relationship between clotting time (minutes) and S.N.F. concentrations. (animal proteinase)

Table 8. Analysis of the milk sample

Determination of total solids			Determination of fat			Determination of S.N.F.	
Dish + solids	17.4248	17.9296	Dish + fat	36.6475	36.8878	I	II
Wt. of dish	17.1832	17.6892	Wt. of dish	36.2952	36.5366	8.557	8.508
Wt. of solids	0.2416	0.2404	Wt. of fat	0.3523	0.3512	Ave. S.N.F. = 8.5302%	
Wt. of sample	2 grms.	2 grms.	Wt. of sample	10 grms.	10 grms.		
% T. S.	12.08	12.02	% fat	3.523	3.512		

Table 9. Effect of variation of Solids-non-fat in milk on animal proteinase activity.[a]

% S.N.F. in milk	Amount of S.N.F. in 25 ml. of milk	Time of clotting (min.)	Type of curd	K = ET (for proteinase)
8.5	-	1.58	soft curd	7.90
9.0	0.25 grms	1.51	soft curd	7.55
9.5	0.375 grms.	1.47	soft curd	7.35
10.0	0.50 grms.	1.35	soft curd	6.75
10.5	0.625 grms.	1.20	soft curd	6.00
11.0	0.750 grms.	1.15	semi-hard	5.75
11.5	0.875 grms.	1.10	semi-hard	5.50
12.0	1.00 grms.	0.67	semi-hard	3.35
12.5	1.125 grms.	0.88	semi-hard	4.40
13.0	1.250 grms.	0.90	hard	4.50
13.5	1.375 grms.	1.00	hard	5.00
14.0	1.500 grms.	1.06	hard	5.30

[a]Samples were taken with 10 cc. of milk, 5 mg. of enzyme, and 0.3 ml. of rennet.

proteinase and casein, different authors reached similar views in different ways.

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} E - S \xrightarrow{K_3} E + P$$

It is assumed that each casein molecule, before it is decomposed, first combines with the proteinase enzyme. Later, after a definite time interval, the substrate is split and throws off its products. In the case of proteinase and casein, different products like proteoses, peptones, polypeptides, and aminocide can be formed. After the products are ejected, the place of combination on the enzyme molecule is left vacant until another casein molecule makes contact.

Combination and decomposition are repeated. The time required for a single cylce is the sum of the time required for another casein molecule to hit the proteinase on the combining point plus the time the proteinase then takes to split the casein molecule and eject its products. The more abundant the casein molecules are about the proteinase enzyme, the shorter will be the probable path of the next casein molecule to the combining point on the proteinase, and hence the shorter will be the average time interval during which the proteinase is left uncombined and therefore inactive, if the casein concentration is great enough. This was done by increasing the concentrations of S.N.F. in the milk; then the inactive interval becomes negligible compared with the interval required for decomposition, when the casein concentrations are at or above the level. The proteinase works at the full speed, because its unused intervals are negligible and further increase of casein concentrations cannot push the reaction rate any faster.

Quantitative formulation of the kinetics of the two-phase reaction in the case of proteinase during clotting

The time required for the cycle can be expressed as the sum of the intervals required for the two consecutive phases.

$$\text{Time required for one cycle} = \frac{1}{K_c S} + K_D \quad - - - \quad (1)$$

S = concentration of the casein

K_c = velocity constant for combination of proteinase and casein

K_D = velocity constant for decomposition of the combined caseinate

$\frac{-ds}{dt}$ = velocity of casein or proteolysis

$\frac{-ds}{dt} = \frac{K}{\text{time}}$ required for 1 cycle

or

$$\frac{-ds}{dt} = \frac{1}{\frac{1}{K_C S} + \frac{1}{K_D}} \quad - - - - - (2)$$

When the concentrations of casein are large enough so that $\frac{1}{K_C S}$ (i.e., when the combination of proteinase and casein is practically instantaneous in comparison with the decomposition phase) the above velocity equation simplifies to

$$---\frac{ds}{dt} = K_D \quad - - - - - (3)$$

Hence, K_D can be determined very simply as the rate of proteolysis in sufficient concentration of casein.

Now, integration of equation (2) gives as the time curve of a single reaction

$$T = \frac{1}{K_C} \log \frac{1}{A - S} + \frac{S}{K_D}$$

where A is the initial casein concentration and S is the concentration after the proteolysis has taken for T minutes. It is obvious that S is so small that S/K_D is negligible and the equation simplifies to the monomolecular reaction

$$T = \frac{1}{K_C} \log \frac{1}{A - S}$$

Table 10. Effect of variation of temperature on the animal proteinase activity.[a]

Temp. in ^{o} C.	Amount of enzyme	Amount of rennet	Clotting time in minutes	Type of curd	K=ET
10^{o}C.	5 mg.	0.3 ml.	2.55	too soft	12.75
20^{o}C.	5 mg.	0.3 ml.	2.50	too soft	12.50
25^{o}C.	5 mg.	0.3 ml.	2.50	too soft	12.50
30^{o}C.	5 mg.	0.3 ml.	1.80	soft	9.00
35^{o}C.	5 mg.	0.3 ml.	1.68	good	8.40
37^{o}C.	5 mg.	0.3 ml.	1.60	good	8.00
40^{o}C.	5 mg.	0.3 ml.	1.51	good	7.55
45^{o}C.	5 mg.	0.3 ml.	1.30	slty. stiff	6.50
50^{o}C.	5 mg.	0.3 ml.	0.86	semi-hard	4.30
55^{o}C.	5 mg.	0.3 ml.	1.70	semi-hard	3.50
60^{o}C.	5 mg.	0.3 ml.	0.65	hard-wheyed off	3.25
65^{o}C.	5 mg.	0.3 ml.	0.61	"	3.05
70^{o}C.	5 mg.	0.3 ml.	0.50	"	2.50

[a]The optimum temp. seems to be at 37^{o}C. At lower temperatures the curd was soft while at temperatures the curd was rubbery.

Table 11. Effect of variation of pH on the animal proteinase activity.[a]

Amount of milk	pH	Amount of enzyme	Amount of rennet	Clotting time in minutes	Type of curd	K = ET
10 ml.	1.5	5 mg.	0.3 ml.	none	----	----
10 ml.	2.0	5 mg.	0.3 ml.	none	----	----
10 ml.	7.0	5 mg.	0.3 ml.	1.87	soft	9.35
10 ml.	7.5	5 mg.	0.3 ml.	1.81	soft	9.08
10 ml.	8.0	5 mg.	0.3 ml.	1.79	good	8.95
10 ml.	8.5	5 mg.	0.3 ml.	1.68	good	8.40
10 ml.	9.0	5 mg.	0.3 ml.	none	----	----
10 ml.	9.5	5 mg.	0.3 ml.	none	----	----

[a]The pH of the milk can be changed by using different buffers. The pH was changed by using 0.1n NaoH on the alkaline side and using pure lactic acid on the acid side. Milk of different pH was tried and observed the effect of animal proteinase on clotting.

It was found that when pH 1.5 or 2.0 were used, no clotting took place even after a long time. The effect of pH 2-6 could not be observed because of the isoelectric point of casein. While on the alkaline side up to pH 8.5 it gave fairly significant results. The milk did not clot at the pH9 or above. It seems that proteinase activity can be best measured at pH between 8-8.5.

Effect of Variations of Different Salts on the Animal Proteinase Activity

In determining the activity of the proteinase, it was necessary to find the effect of different salts, as some salts activate and other salts inhibit the proteinase activity. One percent solution of the following salts were prepared in distilled water and used in 10 ml. of milk samples:

(1)	Potassium Phosphate	KH_2PO_4
(2)	Sodium carbonate	Na_2CO_3
(3)	Calcium chloride	Cacl2
(4)	Sodium citrate	$Na_3C_6H_5O_7 - 2H_2O$
(5)	Ammonium sulphate	$(NH_4)_2SO_4$
(6)	Manganese chloride	MnCl2

Table 12. Effect of potassium phosphate on animal proteinase activity.[a]

Amount of potassium phosphate	Amount of enzyme	Amount of rennet	Time of clotting (min.)	Type of curd	K = ET
1 mg.	5 mg.	0.3 ml.	1.81	soft	9.05
2 mg.	5 mg.	0.3 ml.	1.72	soft	8.60
3 mg.	5 mg.	0.3 ml.	1.20	soft	6.00
4 mg.	5 mg.	0.3 ml.	0.71	good	3.55
5 mg.	5 mg.	0.3 ml.	0.50	good	2.50
6 mg.	5 mg.	0.3 ml.	0.47	semi-hard	2.35
7 mg.	5 mg.	0.3 ml.	0.41	semi-hard	2.05
8 mg.	5 mg.	0.3 ml.	0.37	hard	1.85
9 mg.	5 mg.	0.3 ml.	0.25	hard	1.25
10 mg.	5 mg.	0.3 ml.	0.20	hard	1.00

[a]Potassium phosphate increased the proteinase activity. The optimum concentration of potassium phosphate was between 4-5 mg.

Table 13. Effect of sodium carbonate on animal proteinase activity.[a]

Amount of sodium carbonate	Amount of enzyme	Amount of rennet	Time of clotting (min.)	Type of curd	K = ET
1 mg.	5 mg.	0.3 ml.	0.55	very slight clotting	2.75
2 mg.	5 mg.	0.3 ml.	0.75	very slight	3.75
3 mg.	5 mg.	0.3 ml.	1.10	very slight	5.50
4 mg.	5 mg.	0.3 ml.	none	-	-
5 mg.	5 mg.	0.3 ml.	none	-	-
6 mg.	5 mg.	0.3 ml.	none	-	-
7 mg.	5 mg.	0.3 ml.	none	-	-
8 mg.	5 mg.	0.3 ml.	none	-	-
9 mg.	5 mg.	0.3 ml.	none	-	-
10 mg.	5 mg.	0.3 ml.	none	-	-

[a]The amount of Na_2CO_3 had a direct effect on the proteinase activity. As the concentration of Na_2CO_3 was increased the clotting time was increased and, when the concentration reached 6 mg. or above, there was no clotting of milk.

Table 14. Effect of calcium chloride on the animal proteinase activity.[a]

Amount of calcium	Amount of enzyme	Amount of rennet	Time of clotting (min.)	Type of curd	K - ET
1 mg.	5 mg.	0.3 ml.	0.78	soft	3.90
2 mg.	5 mg.	0.3 ml.	0.62	soft	3.10
3 mg.	5 mg.	0.3 ml.	0.58	good	2.90
4 mg.	5 mg.	0.3 ml.	0.42	good	2.10
5 mg.	5 mg.	0.3 ml.	0.42	good	2.10
6 mg.	5 mg.	0.3 ml.	0.40	firm	2.00
7 mg.	5 mg.	0.3 ml.	0.38	firm	1.90
8 mg.	5 mg.	0.3 ml.	0.31	hard	1.55
9 mg.	5 mg.	0.3 ml.	0.28	hard	1.40
10 mg.	5 mg.	0.3 ml.	0.21	hard	1.05

[a]The increase in CaCl2 concentration increased the proteinase activity. The increased calcium ions made the curd firmer. After certain concentration of the CaCl2, the curd became hard.

Table 15. Effect of manganous carbonate on the animal proteinase activity.[a]

Amount of manganous carbonate	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
1 mg.	5 mg.	0.3 ml.	0/57	semi-liquid	2.85
2 mg.	5 mg.	0.3 ml.	0.51	semi-liquid	2.55
3 mg.	5 mg.	0.3 ml.	0.49	semi-liquid	2.45
4 mg.	5 mg.	0.3 ml.	0.49	semi-liquid	2.45
5 mg.	5 mg.	0.3 ml.	0.38	semi-liquid	1.90
6 mg.	5 mg.	0.3 ml.	0.29	soft	1.45
7 mg.	5 mg.	0.3 ml.	0.26	soft	1.30
8 mg.	5 mg.	0.3 ml.	0.20	soft	1.00
9 mg.	5 mg.	0.3 ml.	0.18	soft	0.90
10 mg.	5 mg.	0.3 ml.	0.12	soft	0.60

[a]The increased concentrations of $MnCO_3$ increased the proteinase activity when it was used less than 5 mg. It did clot the milk.

When different concentrations of Na-Citrate were used in milk samples, the clotting was prevented. In other words, the proteinase activity was inhibited.

Table 16. Effect of ammonium sulfate on animal proteinase activity.[a]

Amount of $(NH_4)So_4$	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
1 mg.	5 mg.	0.3 ml.	1.75	semi-soft	8.74
2 mg.	5 mg.	0.3 ml.	1.62	semi-soft	8.10
3 mg.	5 mg.	0.3 ml.	1.60	semi-soft	8.00
4 mg.	5 mg.	0.3 ml.	1.57	semi-soft	7.85
5 mg.	5 mg.	0.3 ml.	1.55	soft	7.75
6 mg.	5 mg.	0.3 ml.	1.48	soft	7.40
7 mg.	5 mg.	0.3 ml.	1.30	soft	6.50
8 mg.	5 mg.	0.3 ml.	1.21	soft	6.05
9 mg.	5 mg.	0.3 ml.	1.15	firm	5.75
10 mg.	5 mg.	0.3 ml.	1.10	firm	5.50

[a]The increase in concentration of ammonium sulfate decreased the clotting time and hence increased the proteinase activity.

Table 17. Effect of manganese chloride on animal proteinase activity.[a]

Amount of $Mncl_2$	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
1 mg.	5 mg.	0.3 ml.	0.73	soft	3.65
2 mg.	5 mg.	0.3 ml.	0.68	soft	3.40
3 mg.	5 mg.	0.3 ml.	0.62	soft	3.10
4 mg.	5 mg.	0.3 ml.	0.56	semi-hard	2.80
5 mg.	5 mg.	0.3 ml.	0.51	semi-hard	2.55
6 mg.	5 mg.	0.3 ml.	0.46	semi-hard	2.30
7 mg.	5 mg.	0.3 ml.	0.39	hard	1.95
8 mg.	5 mg.	0.3 ml.	0.30	hard	1.50
9 mg.	5 mg.	0.3 ml.	0.22	hard	1.10
10 mg.	5 mg.	0.3 ml.	0.18	hard	0.90

[a]The increase in concentration of $Mncl_2$ decreased the clotting time and hence increased the proteinase activity. The curd was comparatively harder than other curds.

It was determined from the above tables and figure 3, salts like potassium phosphate, CaCl2, $MnCo_3$, ammonium sulfate, decreased the clotting time and hence they increased the proteinase activity; while salts like Na_2Co_3, Na-Citrate increased the clotting time, that is, they decreased the proteinase activity.

Activity of the Plant Proteinase

The effect of various concentrations of the plant proteinase was used to determine its activity. It was found that when 1 gram of the proteinase was dissolved in 100 cc. of distilled water it gave the best results. When 2 percent enzyme solution was used, the curd obtained was too soft and when less than 1 percent proteinase concentration was used the clotting time was prolonged.

Preparation of enzyme solution

One gram of active plant proteinase was dissolved in 100 cc. of distilled water, and the solution was centrifuged at 1000 rpm for 15 minutes and the sediment was removed. The supernatent enzyme solution was used for the tests.

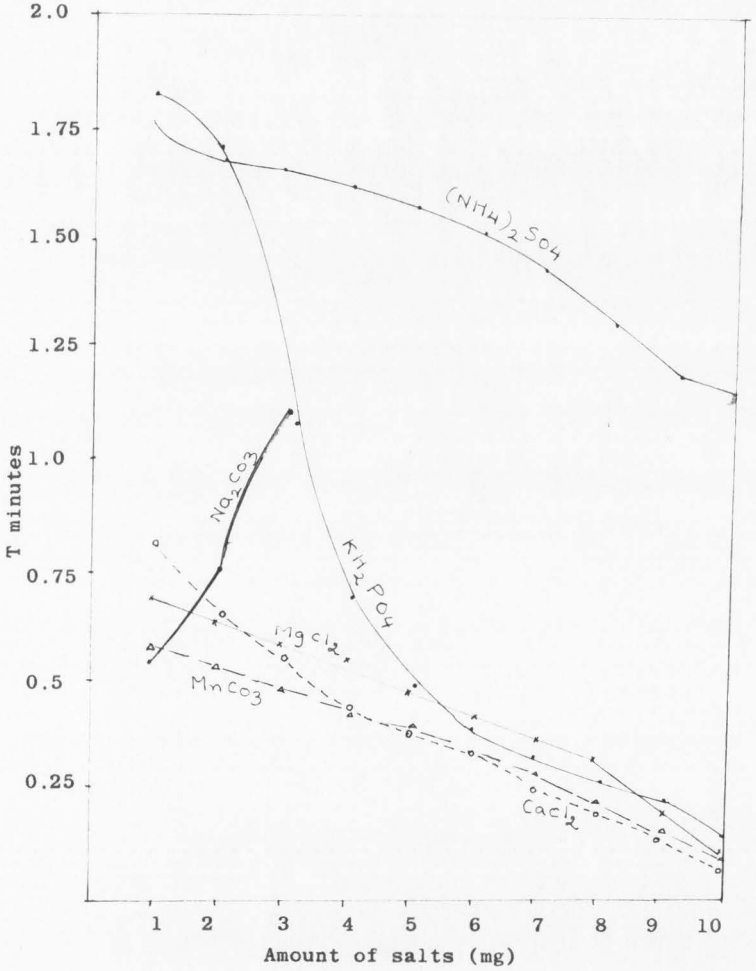


Figure 3. Relationship between clotting time (minutes) and different salts. (Animal proteinase)

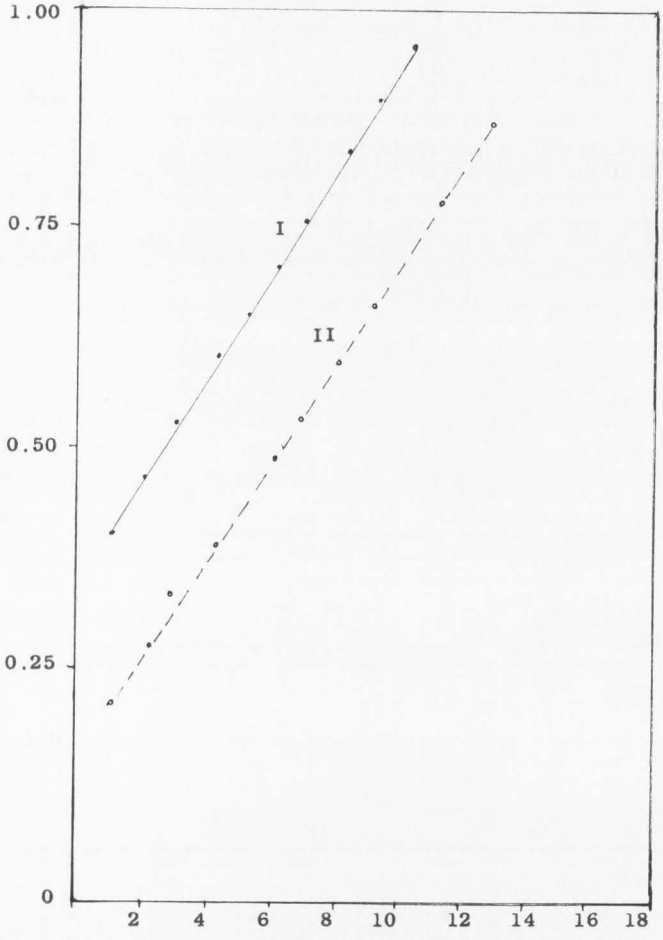


Figure 4. Relation between clotting time (reciprocal) and the amount of enzyme. Curve I represents vegetable proteinase. Curve II represents animal proteinase.

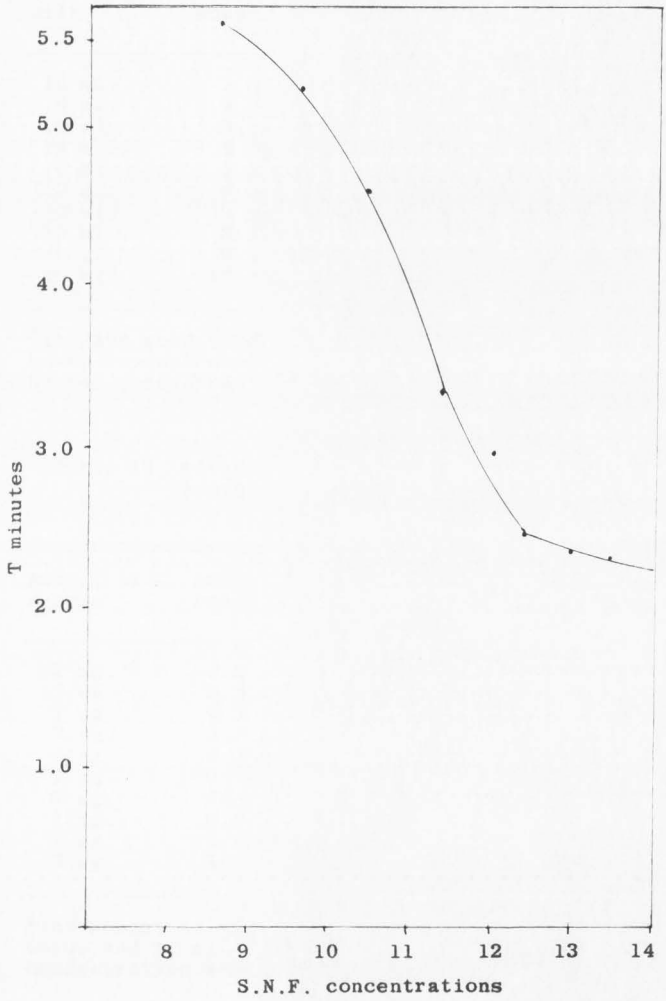


Figure 5. Relationship between clotting time (minutes) and S.N.F. concentrations. (vegetable proteinase)

Table 18. Effect of variation of plant proteinase.[a]

Amount of milk	Amount of enzyme	Clotting time (min.)	Type of curd	K = ET
10 ml.	1 mg.	2.4	soft	2.4
10 ml.	2 mg.	2.0	soft	4.0
10 ml.	3 mg.	1.74	soft	5.22
10 ml.	4 mg.	1.60	soft	6.40
10 ml.	5 mg.	1.56	soft	7.80
10 ml.	6 mg.	1.48	soft	8.88
10 ml.	7 mg.	1.38	good	9.66
10 ml.	8 mg.	1.20	good	9.60
10 ml.	9 mg.	1.10	slight hard	9.90
10 ml.	10 mg.	1.07	slight hard	10.7

[a]It gave good curd at the concentrations between 7-8 mg. Lower concentrations than these gave very soft curd and higher concentrations gave hard curd.

Table 19. Effect of variation of rennet on plant proteinase activity

Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
7 mg.	0.1 ml.	1.55	good	10.85
7 mg.	0.2 ml.	1.30	good	9.10
7 mg.	0.3 ml.	1.20	semi-hard	8.40
7 mg.	0.4 ml.	1.17	semi-hard	8.19
7 mg.	0.5 ml.	1.10	semi-hard	7.70
7 mg.	0.6 ml.	1.05	hard	7.35
7 mg.	0.7 ml.	1.03	hard	7.21
7 mg.	0.8 ml.	0.45	hard	3.15
7 mg.	0.9 ml.	0.43	hard	3.01
7 mg.	1.0 ml.	0.30	hard	2.10

[a]The rennet at the concentration of 0.1 gave higher K value and it also gave good curd. The optimum rennet concentration was taken as 0.1.

Table 20. Effect of variation of solids-not-fat on the plant proteinase activity.[a]

% S.N.F. in milk	Amount of SNF added in 25 ml. of milk	Clotting Time (Min.)	Type of curd	K = ET
8.5	0.25 grms.	5.58	soft	39.06
9.0	0.375 grms.	5.47	soft	38.29
9.5	0.50 grms	5.20	soft	36.40
10.0	0.625 grms.	4.81	soft	33.67
10.4	0.750 grms.	4.61	soft	32.27
11.0	0.875 grms.	3.48	semi-hard	24.36
12.0	1.00 grms.	3.15	semi-hard	22.05
12.5	1.125 grms.	3.0	hard	21.00
13.0	1.250 grms.	2.48	hard	17.36
13.5	1.375 grms.	2.45	hard	17.15
13.5	1.500 grms.	2.31	hard	16.17
14.0		2.11	hard	14.77

[a]Samples were taken with 10 ml. of milk, 7 mg. of enzyme, and 0.1 ml. of rennet. It was determined that as the S.N.F. in the milk was increased, the clotting time decreased, and hence proteinase activity was increased.

Table 21. Effect of variation of temperature on the plant proteinase activity.

Temp. in ^{o} C.	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
10^{o}C.	7 mg.	0.1 ml.	8.30	too soft	58.10
15^{o}C.	7 mg.	0.1 ml.	7.45	too soft	52.15
20^{o}C.	7 mg.	0.1 ml.	7.30	too soft	51.10
25^{o}C.	7 mg.	0.1 ml.	6.48	too soft	45.36
30^{o}C.	7 mg.	0.1 ml.	5.58	soft	39.36
35^{o}C.	7 mg.	0.1 ml.	5.45	soft	39.25
37^{o}C.	7 mg.	0.1 ml.	4.42	soft	36.94
40^{o}C.	7 mg.	0.1 ml.	4.18	good	29.26
45^{o}C.	7 mg.	0.1 ml.	3.50	slight stiff	24.50
50^{o}C.	7 mg.	0.1 ml.	3.10	slight stiff	21.70
55^{o}C.	7 mg.	0.1 ml.	3.05	hard	21.35
60^{o}C.	7 mg.	0.1 ml.	2.65	hard	18.55
65^{o}C.	7 mg.	0.1 ml.	2.15	hard	15.05
70^{o}C.	7 mg.	0.1 ml.	1.35	hard	9.45

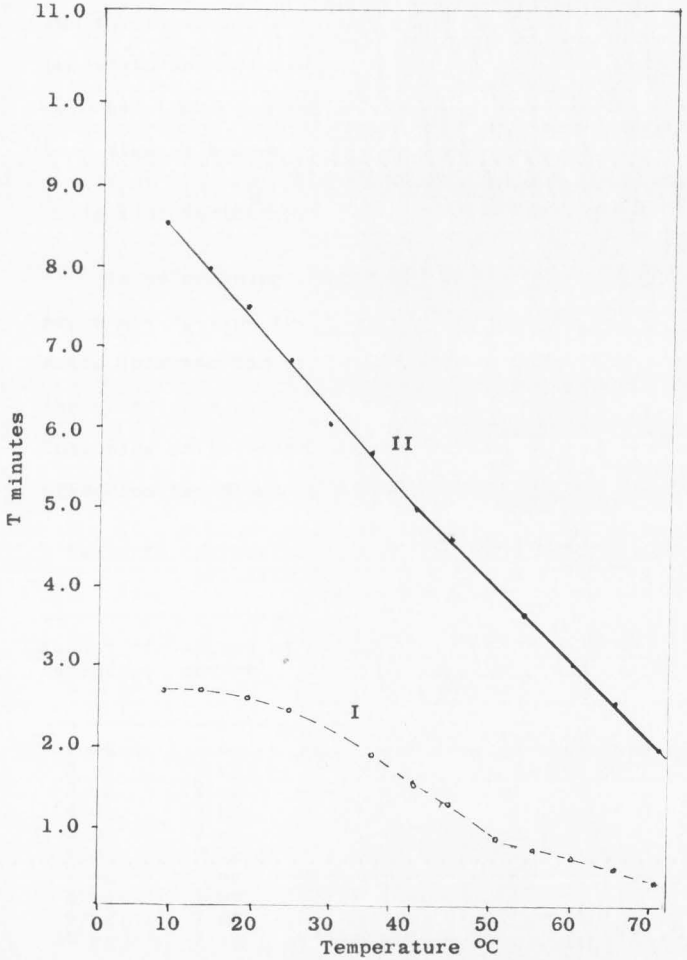


Figure 6. Relationship between clotting time (minutes) and temperature (°C). Curve I represents animal proteinase. Curve II represents vegetable proteinase.

Various temperatures from 10° C to 70° C were tried. The optimum temperature of the plant proteinase activity was at 40° C. At lower temperatures it gave soft curd and the time of clotting was delayed. When the higher temperatures were used the plant proteinase gave hard curd but there was too much separation of whey. The clotting time at higher temperatures was decreased.

Effect of Various Salts on the Plant Proteinase Activity

In determining the plant proteinase activity it was necessary to find the effect of various salts, as some salts increase the proteinase activity while some decrease the proteinase activity. One percent solution of the following salts were prepared in distilled water and their effect on the plant proteinase activity was determined.

Table 22. Effect of potassium phosphate on the plant proteinase activity.[a]

Amount of phosphate	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K =ET
1 mg.	7 mg.	0.1 ml.	4.48	soft	31.36
2 mg.	7 mg.	0.1 ml.	3.05	soft	21.35
3 mg.	7 mg.	0.1 ml.	3.00	soft	21.00
4 mg.	7 mg.	0.1 ml.	2.35	soft	16.45
5 mg.	7 mg.	0.1 ml.	2.30	soft	16.10
6 mg.	7 mg.	0.1 ml.	2.15	soft	15.05
7 mg.	7 mg.	0.1 ml.	2.00	soft	14.00
8 mg.	7 mg.	0.1 ml.	1.55	soft	10.95
9 mg.	7 mg.	0.1 ml.	1.48	soft	10.36
10 mg.	7 mg.	0.1 ml.	1.30	hard	9.10

[a]As the concentrations of KH_2PO_4 was increased the time of clotting was decreased and hence the plant proteinase activity was increased.

Effect of sodium carbonate on the plant proteinase activity

Various concentrations of sodium carbonate were tried but there was no clotting and hence the Na_2Co_3 prevents the proteinase activity.

Table 23. Effect of managanous carbonate on the plant proteinase activity.[a]

Amount of MnCo3	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
1 mg.	7 mg.	0.1 ml.	4.15	soft	29.05
2 mg.	7 mg.	0.1 ml.	4.10	soft	28.70
3 mg.	7 mg.	0.1 ml.	4.00	soft	28.00
4 mg.	7 mg.	0.1 ml.	3.58	soft	25.06
5 mg.	7 mg.	0.1 ml.	3.52	soft	24.64
6 mg.	7 mg.	0.1 ml.	3.00	soft	21.00
7 mg.	7 mg.	0.1 ml.	2.57	soft	17.99
8 mg.	7 mg.	0.1 ml.	2.41	soft	16.87
9 mg.	7 mg.	0.1 ml.	2.38	soft	16.66
10 mg.	7 mg.	0.1 ml.	2.23	soft	15.61

[a]Increase in concentrations of $MnCo_3$ decreased the clotting time and hence increased the plant proteinase activity.

Table 24. Effect of calcium chloride on the plant proteinase activity.[a]

Amount of $Cacl_2$	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K=ET
1 mg.	7 mg.	0.1 ml.	4.45	soft	31.15
2 mg.	7 mg.	0.1 ml.	4.31	soft	30.17
3 mg.	7 mg.	0.1 ml.	4.26	soft	29.82
4 mg.	7 mg.	0.1 ml.	3.57	soft	24.99
5 mg.	7 mg.	0.1 ml.	3.48	soft	24.36
6 mg.	7 mg.	0.1 ml.	3.38	semi-hard	23.66
7 mg.	7 mg.	0.1 ml.	2.55	semi-hard	17.85
8 mg.	7 mg.	0.1 ml.	2.30	semi-hard	16.10
9 mg.	7 mg.	0.1 ml.	2.25	hard	15.75
10 mg.	7 mg.	0.1 ml.	2.21	hard	15.47

[a]When the calcium chloride concentration was increased, there was a decrease in the clotting time, hence the proteinase activity was increased.

Effect of sodium citrate on the plant proteinase activity

Different concentrations of Na Citrate were used, but there was no clotting of the milk. This indicates that Na Citrate inhibits the proteinase activity.

Table 25. Effect of ammonium sulfate on the plant proteinase activity

Amount of ammonium sulfate	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K = ET
1 mg.	7 mg.	0.1 ml.	6.31	soft	44.17
2 mg.	7 mg.	0.1 ml.	6.17	soft	43.19
3 mg.	7 mg.	0.1 ml.	6.03	soft	42.21
4 mg.	7 mg.	0.1 ml.	5.59	soft	39.13
5 mg.	7 mg.	0.1 ml.	5.30	soft	37.10
6 mg.	7 mg.	0.1 ml.	5.15	hard	36.05
7 mg.	7 mg.	0.1 ml.	5.00	hard	35.00
8 mg.	7 mg.	0.1 ml.	4.80	hard	33.60
9 mg.	7 mg.	0.1 ml.	4.35	hard	30.45
10 mg.	7 mg.	0.1 ml.	3.20	hard	22.40

It was determined from the above tables and figure 7 that salts like potassium phosphate, calcium, chloride, manganous carbonate and ammonium sulfate, decreased the clotting time and hence there was increased in the vegetable proteinase activity, while salts like sodium carbonate, sodium citrate prevented clotting.

Effect of pH

The effect of pH on the vegetable proteinase activity was determined by changing the pH of milk on alkaline ssd side with 1 N NaOH. On the acid side, the pH of the milk was changed with the help of pure lactic acid.

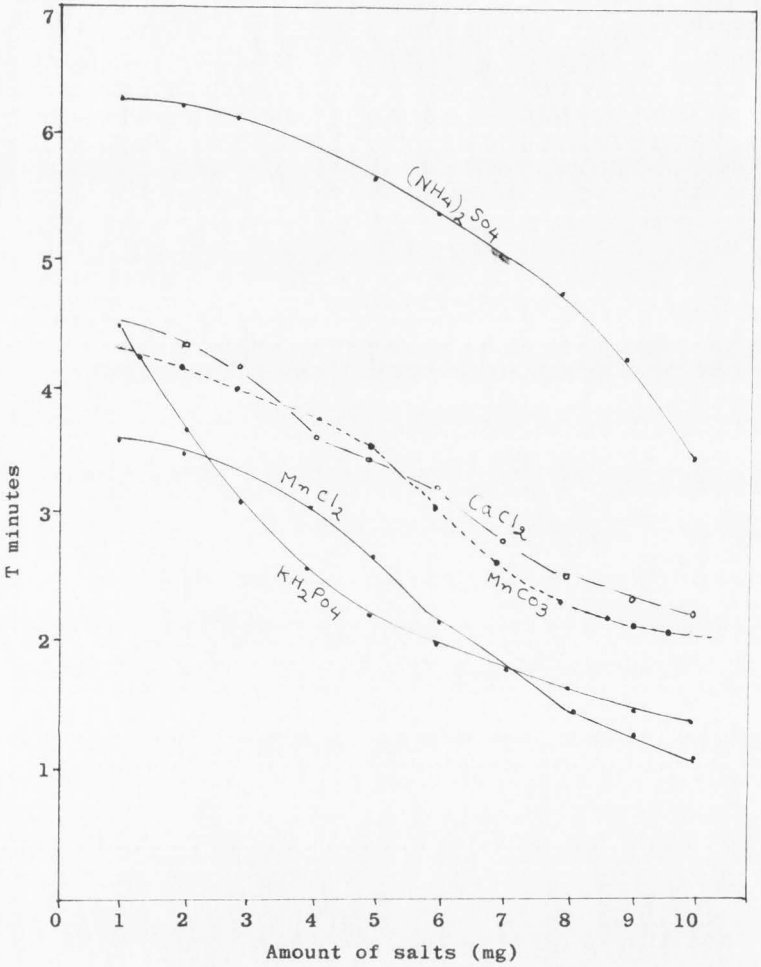


Figure 7. Relationship between clotting time (minutes) and different salts.(vegetable proteinase)

It seems that the optimum pH of the vegetable proteinase is between 7-7.5.

Table 26. Effect of pH on the plant proteinase activity

Amount of milk	pH	Amount of enzyme	Amount of rennet	Clotting time (min.)	Type of curd	K=ET
10 ml.	1.0	7 mg.	0.1 ml.	none	----	-----
10 ml.	3.0	7 mg.	0.1 ml.	none	----	-----
10 ml.	7.0	7 mg.	0.1 ml.	4.30	soft	29.10
10 ml.	7.5	7 mg.	0.1 ml.	5.30	soft	37.10
10 ml.	8.0	7 mg.	0.1 ml.	5.67	soft	39.69
10 ml.	8.5	7 mg.	0.1 ml.	none	----	-----
10 ml.	9.0	7 mg.	0.1 ml.	none	----	-----
10 ml.	9.5	7 mg.	0.1 ml.	none	----	-----
10 ml.	10.0	7 mg.	0.1 ml.	none	----	-----
10 ml.	10.5	7 mg.	0.1 ml.	none	----	-----

Table 27. Animal proteinase activity by A.O.A.C. method

Amount of enzyme	Burrette reading before incubation in cc.	Burette reading after incubation in cc.	Difference in cc.	Activity in units/mg.
1 mg.	0.5	0.7	0.2	0.2
2 mg.	0.4	0.8	0.4	0.4
3 mg.	0.4	0.9	0.5	0.5
4 mg.	0.3	1.0	0.7	0.7
5 mg.	0.6	1.2	0.6	0.6
6 mg.	0.5	1.3	0.8	0.8
7 mg.	0.4	1.33	0.93	0.93
8 mg.	0.41	1.4	0.99	0.99
9 mg.	0.6	1.6	1.00	1.00
10 mg.	0.72	1.75	1.03	1.03

Table 28. Vegetable proteinase activity by A.O.A.C. method

Amount of enzyme	Burette reading before incubation in cc.	Burette reading after incubation in cc.	Difference in cc.	Activity in units/mg.
1 mg.	1.1	1.3	0.3	0.3
2 mg.	1.1	1.4	0.3	0.3
3 mg.	1.2	1.6	0.4	0.4
4 mg.	1.34	1.74	0.4	0.4
5 mg.	1.5	2.01	0.51	0.51
6 mg.	1.8	2.34	0.54	0.54
7 mg.	1.9	2.50	0.60	0.60
8 mg.	2.0	2.62	0.62	0.62
9 mg.	2.1	2.82	0.73	0.73
10 mg.	2.2	2.97	0.77	0.77

The animal proteinase was more active than the vegetable proteinase.

Comparisons of Bitterness of the Curd Developed by Animal and Vegetable Proteinases

An experiment was run to determine the bitterness of the curd by using different concentrations of animal and vegetable proteinases.

Animal proteinase

Different concentrations like 0.5 to 25 mg. of animal proteinase were tried and tested for bitterness. It was found that there was no bitterness in the lower concentrations. The curd was sweet. There was developed a very slight bitterness at the concentration of 22 mg. of

animal proteinase and the bitterness was increased in the higher concentrations. When 27 mg. or above concentrations of animal proteinase were used there was no clotting.

Vegetable proteinase

In the case of vegetable proteinase different concentrations like 0.5 mg. to 25 mg. of 0.5 percent, 1 percent, and 2 percent were used. It was found that bitterness was developed at the lower concentrations of 3 mg. of 0.5 percent vegetable proteinase, where the clotting time was very much delayed and the intensity of bitterness was increased with the increase in concentration. The curd was very much bitter at the concentration of 5 mg. of 2 percent vegetable proteinase concentration.

It was found that bitterness in the case of animal proteinase developed only at the higher concentrations, while in the case of vegetable proteinase the bitterness was developed at the lower concentrations and the intensity of bitterness was increased with the increase of concentrations. The bitterness might be due to peptone formation causing the breakdown of casein.

Nitrogen Determinations

Total nitrogen can be determined by Kjeldahl method while amino nitrogen can be determined by Van Skyke method. The free carboxyl groups can be determined by Formal titration method. During the study formal titration method was used to determine the free carboxyl groups in

the proteolytic material.

Method

The milk was first heated to 37° C. In the case of animal proteinase enzyme, 5 mg. of enzyme solution and 0.3 ml. of rennet were added while in the case of vegetable proteinase, 7 mg. of enzyme and 0.1 ml of rennet were added. The clotting took place in a few minutes. The tubes were then centrifuged to separate casein from the proteolytic material at 18,000 r.p.m. for 15 minutes. The supernatent material was poured into the separate tubes and was analysed for the nitrogen content.

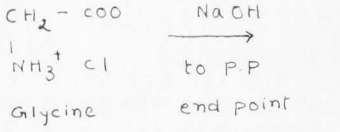
Formal titration method

One ml. of the sample was taken in a 50 ml. Erlenmeyer flask and 2 drops of phenolptalein indicator was added and titrated to a faint pink color with 0.25 N. NAOH. The burette reading was recorded. Then 10 ml. of neutral formalin (40 percent HCHO) was added and titrated again to a definite pink end point. The difference in the burette reading is the formal value.

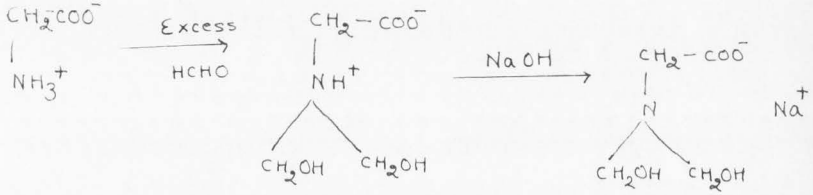
Table 29. Analysis of nitrogen determination

	Amount of enzyme	Amount of rennet	Nitrogen by formal titration method		Average Nit./ml.
			I	II	
1. Animal proteinase	5 mg.	0.3 ml.	36.6	36.2	34.6
2. Vegetable proteinase	7 mg.	0.1 ml.	27.85	27.80	27.82

Reactions During Formal Titration



(only a few % of the NH_3^+ groups neutralized by titrating to pH 8·3)



SUMMARY

During the study three methods were studied, i.e., Fuld Gross Method (A.O.A.C.), Curd Tension Method, and the Clotting Method. The Clotting Method was selected and then modified for practical purposes for determining the proteinase activity.

The modified clotting method for determining animal proteinase activity is as follows: Weigh accurately 0.7 grams of an unactive proteinase and dissolve in 100 cc. of distilled water. Then add 0.3 grams of active enzyme to activate an unactive enzyme. Stir well and centrifuge the enzyme solutions at 1,000 rpm for 15 minutes. The sediment is removed and only the supernatent clear solution is used for the test.

9 cc. of rennet extract is dissolved in 91 cc. of cold water. 10 cc. of milk is heated to 37° C. before using the enzyme. Then add 0.5 ml. (5 mg.) of enzyme solution and 0.3 ml. of rennet solution, and invert the tube 4 times to mix it properly and allow it to stand. Note the time of clotting with the help of a stopwatch. The clotting can be seen by slightly bending the tube.

The modified clotting method for determining the plant proteinase activity is as follows: Weigh accurately 1 gram of the active plant proteinase and dissolve in 100

cc of distilled water. The solution is then centrifuged at 1,000 rpm for 15 minutes. The sediment is removed and the clear supernatent solution is taken for the test.

Nine cc. of rennet extract is dissolved in 91 cc. of cold distilled water. Ten cc. of milk is heated to 40° C. before using the enzyme. Then 0.7 ml. (7 mg.) of enzyme solution is added, followed by 0.1 ml. of rennet solution. Then invert the tube 4 times to mix the enzyme properly. Note the time of clotting with the help of a stopwatch.

CONCLUSION

1. The curd formed by the animal proteinase tasted sweeter at the lower concentrations and slight bitterness developed at the higher concentrations. The curd formed by the plant proteinase tasted bitter at all concentrations. The intensity of bitterness was increased as the concentrations of both the proteinases were increased. The developed bitterness might be due to the peptone formation from the breakdown of casein by the proteolytic enzymes.

2. It was concluded from the experiments that the activity of the animal proteinase is more than that of the plant proteinase.

3. The modified clotting method gave fairly accurate results. The replicate values of the clotting time agreed with each other. It is also a rapid and practical method as compared to other methods.

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